TWEAK can induce cell death via endogenous TNF and TNF receptor 1

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TWEAK is a recently cloned novel member of the TNF ligand family. Here we show that soluble TWEAK is sufficient to induce apoptosis in Kym-1 cells within 18 h. TWEAK-induced apoptosis is indirect and is mediated by the interaction of endogenous TNF and TNF receptor (TNFR)1, as each TNFR1-Fc, neutralizing TNF-specific antibodies and TNFR1-specific Fab fragments efficiently antagonize cell death induction. In addition to this indirect mode of action, co-stimulation of Kym-1 cells with TWEAK enhances TNFR1-mediated cell death induction. In contrast to TNF, TWEAK does only modestly activate NF-κB or c-jun N-terminal kinase (JNK) in Kym-1 cells. Although TWEAK binding to Kym-1 cells is easily detectable by flow cytometric analysis, we found neither evidence for expression of the recently identified TWEAK receptor Apo3/TRAMP/wsl/DR3/LARD, nor indications for direct interactions of TWEAK with TNFR. Together, these characteristics of TWEAK-induced signaling in Kym-1 cells argue for the existence of an additional, still undefined non-death domain-containing TWEAK receptor in Kym-1 cells.

Key words: TWEAK / Endogenous TNF / Apoptosis / Kym-1

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1 Introduction

The members of the TNF ligand family mediate various biological effects including apoptosis, differentiation and inflammatory responses [1, 2]. Most ligands of this family are primarily expressed as type II membrane proteins, but for many of these cytokines, soluble forms can be released by proteolytic processing [2]. These ligands exert their biological functions by binding to one or more members of a complementary family of receptors, which form the TNFR superfamily [1, 2]. This receptor family is characterized by the presence of several cysteine-rich motifs of about 40 amino acids in the extracellular domains [1, 2]. The intracellular domains of these receptors are more heterogeneous and show little sequence similarity, except in a subgroup of the TNFR that induce apoptosis via an intracellular death domain motif [3]. Members of this subgroup are believed to engage the apoptotic machinery via cytoplasmic death domain-containing adaptor molecules like TRADD [4], FADD/MORT [5, 6] and RAIDD [7] that in turn recruit caspases with large regulatory domains like caspase-2, -8 and -10 [7–10]. With regard to signaling mechanisms, the most prominent feature of the other non-death domain-containing members of the TNFR superfamily is their binding to members of the TNFR-associated factor (TRAF) family leading to NF-κB and c-jun N-terminal kinase (JNK) activation [11]. Nevertheless, induction of apoptosis by non-death domain-containing members of the TNFR superfamily has also been described although the underlying mechanisms are still poorly understood [12–15]. TWEAK/Apo3L is a novel member of the TNF ligand family that weakly induces apoptosis, mediates chemokine induction, activates NF-κB and indudcs proliferation in endothelial cells [16–18]. In a recent study, the orphan death domain-containing receptor Apo3/wsl/TRAMP/DR3/LARD [19–23] has been identified as a receptor for TWEAK [17]. Here, we show that TWEAK-induced apoptosis critically depends on the autocrine/paracrine action of induced TNF. Moreover, as the response pattern of TWEAK in Kym-1 cells is quite distinct from that of TNF and as we have obtained no evidence for Apo3/TRAMP expression, we propose the existence of another TWEAK receptor distinct from Apo3/TRAMP.

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Abbreviations: EMSA: Electrophoretic mobility shift assay **JNK:** c-jun N-terminal kinase **PNGase F:** Peptide N-glycanase F

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2 Results and discussion

2.1 Characterization of recombinant TWEAK

The extracellular domain of human TWEAK was expressed in human embryonic kidney 293 cells as a soluble, FLAG-tagged and N-glycosylated cytokine (sTWEAK). Treatment of sTWEAK with peptide N-glycanase F (PNGaseF) induced a shift in molecular mass from 23 to 19 kDa, indicating that the unique putative N-glycosylation site predicted from the primary sequence of TWEAK is indeed utilized (Fig. 1A). sTWEAK displayed a molecular mass of about 63 kDa when analyzed by gel permeation chromatography (*i. e.* 2.7 times the size of the monomer), suggesting that sTWEAK is a homotrimer under native conditions (Fig. 1B). This is in agreement with crystallographic data demonstrating that other members of the TNF family (TNF, lymphotoxin α and CD40L) are homotrimers [24–26]. Purified sTWEAK was able to induce cell death in the human colon carcinoma cell line HT-29 when added simultaneously with IFN-γ, and this cytotoxicity could not be further increased by cross-linking of sTWEAK ([16, 27] and data not shown). This is in contrast with some other ligands of the TNF family, like FasL and TRAIL, which become up to 1000-fold more active when cross-linked [27]. Taken together, these date indicate that sTWEAK is most probably active as a homotrimer.

2.2 TWEAK induces cell death in Kym-1 cells via endogenous TNF/TNFR1 interaction

We have screened several cell lines for their sensitivity to sTWEAK and found that Kym-1 cells, a rhabdomyosarcoma cell line, was efficiently killed by sTWEAK, whether or not sTWEAK was cross-linked by anti-FLAG antibodies (Fig. 2A). These data indicate that mere trimerization of TWEAK receptors is sufficient for the initiation of the apoptotic program in Kym-1 cells. The kinetics of apoptosis induction by sTWEAK in Kym-1 cells was slower than that induced by TNF, but similar to that observed in the same cells upon triggering of TNFR2 (Fig. 2B). Kym-1 cells are indeed one of the rare cellular models in which apoptosis can be induced by exclusive triggering of each of both TNFR [15]. We have recently found that the apoptotic response initiated by agonistic TNFR2-specific antibodies was based on the induction of expression of endogenous transmembrane TNF that in turn kills Kym-1 cells by acting on TNFR1 via an autocrine/paracrine loop (manuscript in preparation). As a consequence of this indirect mechanism of action, the onset of TNFR2-initiated apoptosis is significantly delayed compared to that mediated by TNFR1 (Fig. 2B). The same delay in the onset of apoptosis was observed when sTWEAK was added to the cells (Fig. 2B). To test whether a similar indirect mechanism of apoptosis could also be initiated by sTWEAK, we included in the assay various agents antagonizing TNF action. sTWEAK-induced apoptosis was completely abrogated by either the extracellular domain of TNFR1 fused to the Fc por-

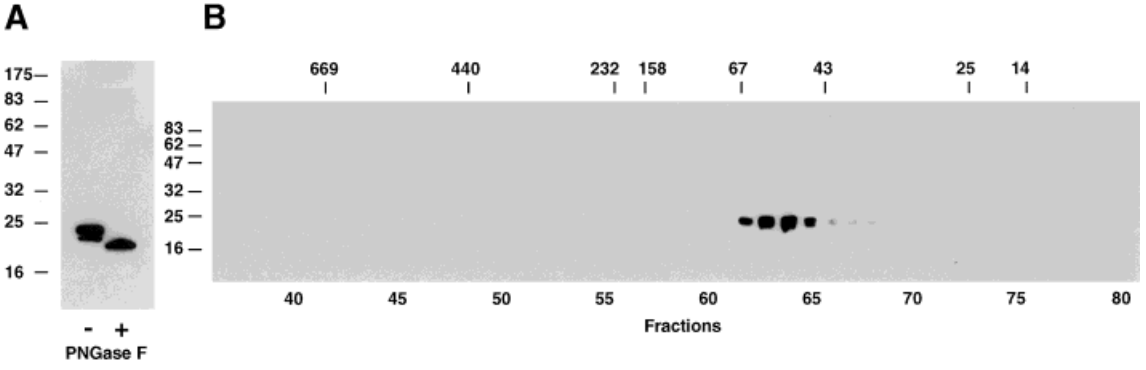

Figure 1. Characterization of recombinant FLAG-tagged soluble TWEAK. (A) SDS-PAGE analysis of purified sTWEAK with and without PNGase F treatment. Molecular masses (kDa) of standards are indicated. (B) Gel filtration analysis of sTWEAK. sTWEAK-containing supernatants were concentrated and loaded onto a Superdex 200 column. Fractions were analyzed for the presence of sTWEAK by Western blotting using the FLAG-specific mAb M2. Elution volumes of various molecular mass standards are indicated.

tion of human IgG1, TNFR1-Fc (but not by TRAIL-R2-Fc; data not shown), TNFR1-specific Fab fragments and neutralizing TNF-specific antibodies (Figs. 2C, D and 3A). This endogenous TNF is probably membrane bound, because the supernatants of sTWEAK-treated Kym-1 cells were non-toxic for the TNF-sensitive WEHI-164 cell line, indicating that no significant amounts of soluble (s)TNF were produced in response to sTWEAK (data not shown). Preliminary data indicate, however, that sTWEAK/IFN-γ killing of HT-29 cells [16] is not inhibited by TNFR1-Fc (data not shown), pointing to the existence of an alternative mechanism of apoptosis induction by sTWEAK. Nevertheless, indirect apoptosis induction via TNF and TNFR1 has also been reported to occur after TNFR2 stimulation and may thus represent a rather general mechanism by which non death-domain receptors trigger apoptosis ([28], manuscript in preparation).

2.3 TWEAK enhances TNFR1-mediated cell death

Triggering of TNFR2 not only leads to TNF induction but also to a marked enhancement of TNFR1-induced apoptosis via an intracellular mechanism which most likely relies on the inactivation of TRAF2, leading to the abrogation of TNFR1-mediated induction of anti-apoptotic factors [29, 30]. We have therefore analyzed whether sTWEAK treatment results in a similar sensitization towards TNFR1-induced apoptosis. For this purpose, the effect of sTWEAK-induced, endogenously produced TNF was neutralized using TNF-specific antibodies. In addition, TNFR1 was stimulated with an agonistic TNFR1-specific antibody at a concentration that induces no or only very limited apoptosis on its own. As shown in Fig. 3B, neither the lowest concentration of the agonistic TNFR1-specific antibody Htr-1 alone nor the combination of sTWEAK and anti-TNF antibodies induced significant cell death in Kym-1 cells. However, when Htr-1 and sTWEAK plus anti-TNF antibodies were applied, a strong

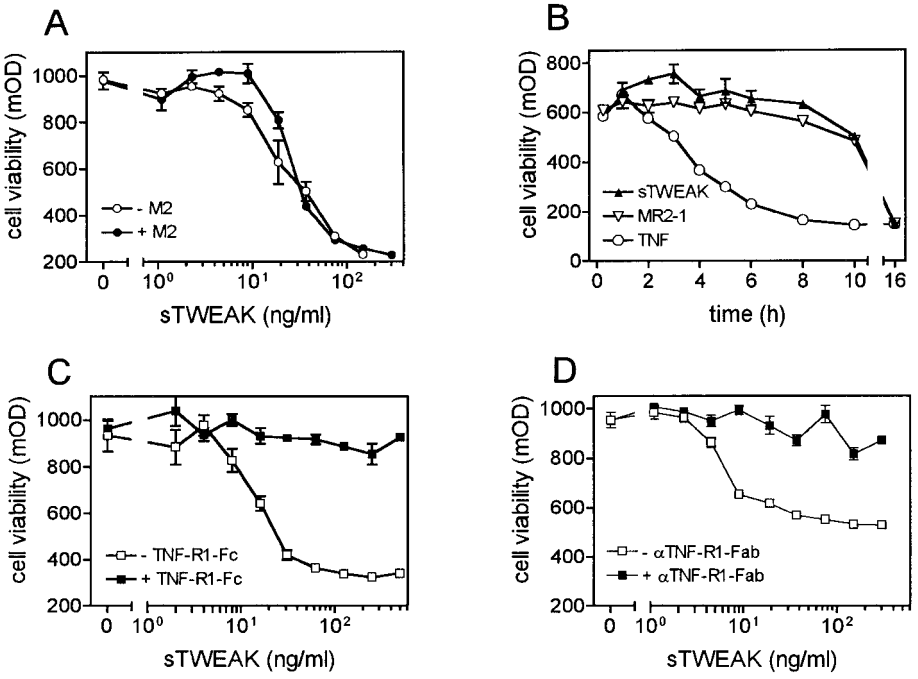

Figure 2. TWEAK induces cell death in Kym-1 cells via endogenous TNF and TNFR1. (A) Kym-1 cells (1×10^4) were cultured overnight in 96-well assay plates. The next day cells were treated with the indicated concentrations of sTWEAK alone (open circles) or complexed with the aggregating antibody M2, cultivated overnight and analyzed for viability using the MTT procedure. (B) Kym-1 cells (1×10^4) were cultured overnight in 96-well assay plates. The next day cells were treated with the indicated concentrations of sTWEAK (100 ng/ml, filled triangles), the agonistic TNFR2-specific antibody MR2-1 (1 μg/ml, open triangles) or TNF (10 ng/ml, open circles) for the indicated time and finally analyzed for viability using the MTT procedure. (C, D) Kym-1 cells were treated with the indicated concentrations of sTWEAK in the presence (filled squares) or absence (open squares) of TNFR1-Fc (C, 5 μg/ml) and TNFR1-specific Fab fragments (D, 20 μg/ml), respectively, and finally analyzed for viability using the MTT procedure.

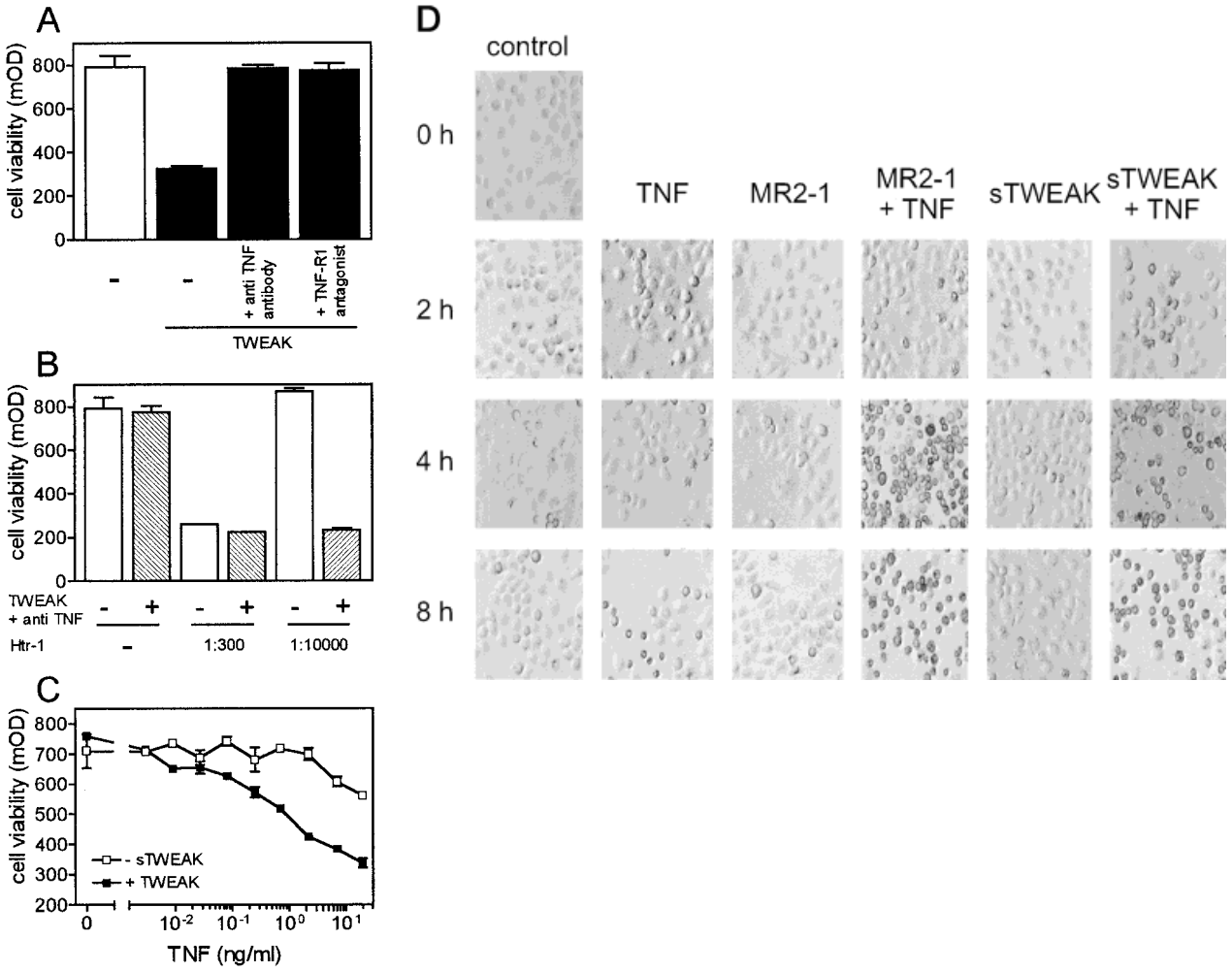

Figure 3. TWEAK enhances TNFR1-mediated cell death in Kym-1 cells. (A) Kym-1 cells were treated with 200 ng/ml sTWEAK in the presence or absence of TNFR1-Fc (5 μg/ml) and neutralizing TNF-specific antibodies (30 μg/ml) and finally analyzed for viability using the MTT procedure. (B) Kym-1 cells were incubated with the indicated concentrations of the TNFR1-specific agonistic mAb Htr-1 in the presence (hatched bars) or absence (open bars) of a non-apoptosis-inducing combination of sTWEAK (100 ng/ml) and neutralizing anti-TNF Ab (30 μg/ml). Cell viability was determined the next day using the MTT procedure. (C) Kym-1 cells were treated with the indicated concentration of TNF in the presence (filled squares) and absence (open squares) of 200 ng/ml sTWEAK. After 4 h cell viability was determined using the MTT procedure. (D) Kym-1 cells were incubated with 1 ng/ml TNF, 200 ng/ml sTWEAK, the TNF-R2-specific mAb MR2-1 (1 μg/ml) or combinations thereof and photographs were taken after the indicated time periods.

apoptotic response could be observed. This observed synergism indicates that the TWEAK receptor, similar to TNFR2, is able to enhance TNFR1-induced apoptosis in Kym-1 cells (Fig. 3B). An enhancement of TNFR1-mediated cell death by co-stimulation with sTWEAK is also shown in a different experimental setting, devoid of TNF-neutralizing reagents. While neither low doses of soluble TNF (1 ng/ml), that almost exclusively trigger TNFR1 [31, 32], nor stimulation of Kym-1 cells with agonistic TNFR2-specific mAb or sTWEAK resulted in significant signs of cell death within 4–8 h after treatment, co-stimulation of TNFR1 together with either TNFR2 or sTWEAK caused pronounced cell death (Fig. 3C, D). We can rule out that sTWEAK acts via binding to TNFR2 as TNFR2-specific Fab fragments had no impact on sTWEAK-mediated apoptosis and because sTWEAK did not bind to TNFR2-Fc (data not shown). Taken together, these results suggest that sTWEAK engages a receptor on Kym-1 cells that is not directly linked to the apoptotic caspase cascade and therefore is most likely distinct from the death domain-containing receptor Apo3/TRAMP. Since Apo3/TRAMP has recently been reported

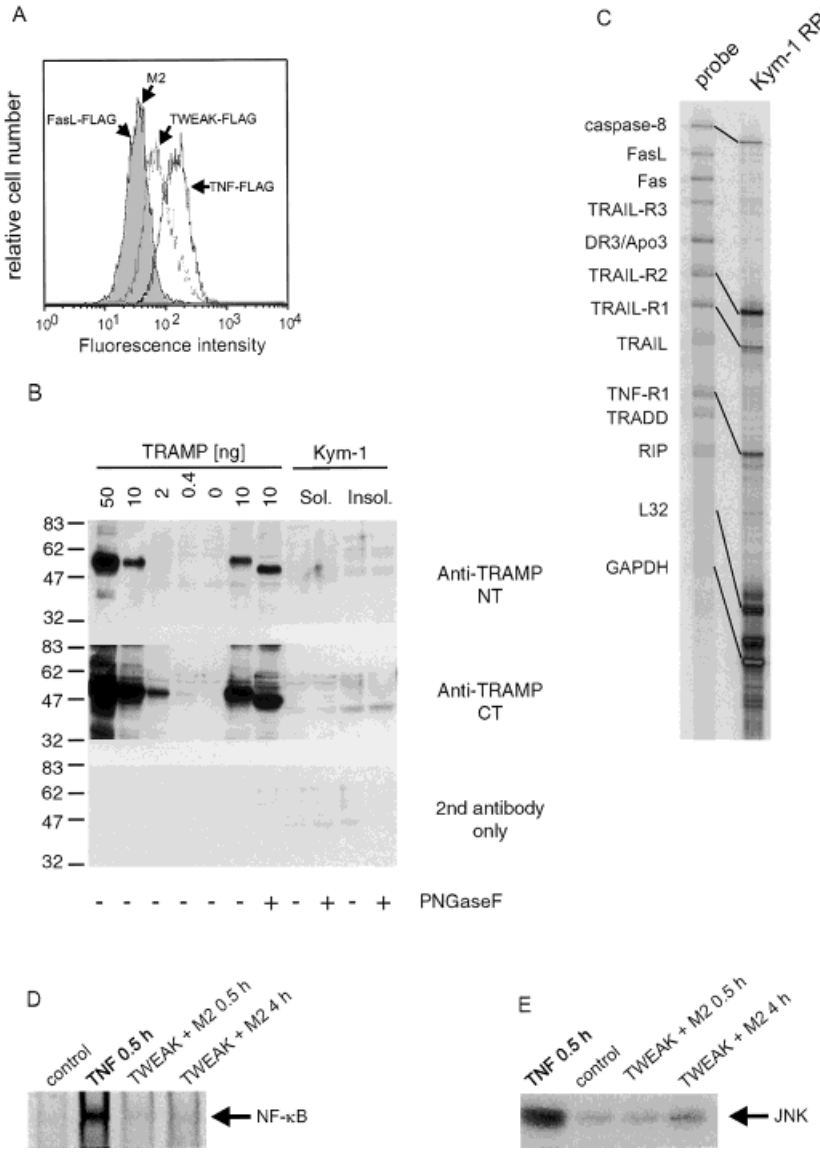

Figure 4. Lack of evidence for Apo3/TRAMP signaling in Kym-1 cells upon TWEAK treatment. (A, B, C) Kym-1 cells bind sTWEAK but show not detectable amounts of Apo3/TRAMP expression. Kym-1 cells were incubated with FLAG-tagged TWEAK and FLAG-tagged Fas ligand (FasL) as a control. After several washes the cells were incubated with the FLAG-specific antibody M2 followed by a FITC-labeled rabbit anti-mouse antisera to detect bound FLAG-tagged ligands (A). (B) Western blot analysis of Apo3/TRAMP expression in Kym-1 cells. Lysate and insoluble pellet of 1×10^5 Kym-1 cells were treated with or without PNGaseF as described in Sect. 4.6 and analyzed for Apo3/TRAMP expression with two different Apo3/TRAMP-specific antisera (anti-TRAMP NT, anti-TRAMP-CT) by Western blotting. As a control extracts of Apo3/TRAMP-transfected 293T cells were diluted in untransfected 293T extracts and analyzed on the same gel. The amounts of TRAMP in these controls had been previously determined on a separate Western blot by comparison with known amounts of TRAMP-Fc (data not shown). (C) RNase protection assay analysis of steady-state levels of various apoptosis-relevant molecules including Apo3/TRAMP. Total RNA (10μg) was analyzed with the hApo-3c Multi-Probe template set to detect caspase-8, FasL, Fas, DcR1, Apo3/DR3/TRAMP, DR4, DR5, TRAIL, TNF-R1, TRADD, RIP and L32 and GAPDH as internal controls. Protected probes were resolved by electrophoresis on a denaturing polyacrylamide gel and quantified by phosphorimaging (B). (D, E) TNF but not sTWEAK is a potent inducer of NF-κB and JNK in Kym-1 cells. Kym-1 cells (1×10^6) were treated with 10 ng/ml TNF or 200 ng/ml sTWEAK for the indicated period of time and were subsequently analyzed for NF-κB activation by EMSA (D) or JNK activity by immunocomplex kinase assays (E).

to bind to TWEAK [17], we next tested whether Kym-1 cells would express this receptor. Although we could demonstrate significant sTWEAK binding on Kym-1 cells by FACS analysis (Fig. 4A), we obtained no indication for Apo3/TRAMP expression in this cell line using either two different Apo3/TRAMP-specific antisera in Western blotting experiments (Fig. 4B) or RNase protection assays (Fig. 4C). Kym-1 cell equivalents (1×10^5) were analyzed by Western blotting. The detection limits of the Apo3/TRAMP-specific antisera were about 0.4 ng (Fig. 4B), corresponding to a detection limit of 1200 TRAMP molecules per cell. Overexpression of Apo3/TRAMP results in a pronounced activation of NF-κB [19–23] and JNK (data not shown) in several cell lines. However, there was no significant response found in this regard in sTWEAK-treated Kym-1 cells (Fig. 4D, E). Taken together these results suggest that Apo3/TRAMP is not expressed in Kym-1 cells.

3 Concluding remarks

We have shown that sTWEAK induces TNF expression in Kym-1 cells and at the same time sensitizes TNFR1 to the cytotoxic effects of TNF. sTWEAK is therefore not cytotoxic on its own in this model but only helps to provide factors allowing for autocrine/paracrine killing of the cells via TNF and TNFR1. Triggering of the TWEAK receptor does not significantly activate NF-κB or JNK pathways. None of these results would have been predicted from what we know so far about the death domain-containing receptor Apo3/TRAMP. Because of these results and because we found no evidence for Apo3/TRAMP expression in Kym-1 cells, we predict that Kym-1 cells express a TWEAK receptor distinct from Apo3/TRAMP that is devoid of an intracellular death domain.

4 Materials and methods

4.1 Antibodies

The TNFR2-specific antibody MR2-1 was a gift from W. Buurman, University of Limburg, Maastricht, The Netherlands. The TNFR1- and TNFR2-specific Fab fragments are derived from the mAb H398 and a polyclonal TNFR2-specific rabbit IgG fraction is described elsewhere [31]. The agonistic TNFR1-specific mAb Htr-1 was a gift from W. Lesslauer, Hoffmann-La Roche Ltd., Basel and TNF was a gift from I.-M. von Broen, Knoll AG, Ludwigshafen, Germany. The anti-human TNF mAb (357-101-4) was provided by A. Meager, National Institute for Biological Standards and Control, Potters Bar, GB. The Apo3/TRAMP-specific polyclonal rabbit serum anti-TRAMP NT (AL104) raised against a peptide comprising amino acid residues 1-23 of the mature protein was described elsewhere [20]. The Apo3/TRAMP-specific polyclonal rabbit serum anti-TRAMP CT raised against a peptide of the C-terminal part of Apo3/TRAMP was from Millenium Biotechnology (Ramona, CA).

4.2 Purification of TWEAK

sTWEAK was produced as described [27]. Briefly, the extracellular domain of human TWEAK (coding for amino acids 106-249) was subcloned in a modified version of the PCR-III vector (Invitrogen, NV Leek, The Netherlands), in frame with the hemagglutinin signal peptide and a FLAG epitope. Conditioned supernatants of stably transfected 293 cells were harvested after 14 days of culture. FLAG-tagged sTWEAK was purified on M2-agarose (Sigma) and eluted in 50 mM citrate-NaOH, pH 2.5, followed by neutralization with Tris-HCl pH 8. The buffer was exchanged to PBS and the protein concentrated in Centrikon-30 concentrators (Amicon Corp., Easton, TX).

4.3 Cell death assays

Kym-1 cells (1×10^4 per well) were cultivated in 96-well microtiter plates overnight. The next day TWEAK-FLAG, TWEAK-FLAG-M2-complex (M2 constant 0.5 μg/ml) and/or antibodies were applied as indicated and after additional 18 h of culture metabolic activity was measured by the 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method.

4.4 EMSA analysis of NF-κB activation

Kym-1 cells (10^6) were seeded in 60-mm cell culture dishes and cultivated overnight. The next day the cells were stimulated for various times with the indicated combinations of TNF and TWEAK. Preparation of nuclear extracts was described elsewhere [29] and electrophoretic mobility shift assays (EMSA) were performed using a standard procedure using a HPLC-purified NF-κB-specific oligonucleotide (5'-ATC AGG GAC TTT CCG CTG GGG ACT TTC CG-3'), end-labeled with [γ-^{32}P]ATP.

4.5 Immune complex JNK assay

Immune complex JNK assays were essentially performed as described elsewhere [33]. In brief, 10^6 Kym-1 cells were seeded in 60-mm cell culture dishes and cultivated overnight. The next day cells were stimulated as indicated, harvested with a rubber policeman, washed once with PBS and lysed in 1 ml of kinase lysis buffer [200 mM Tris, pH 7.4; 5 mM $MgCl_2$, 1 % Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonylfluoride (PMSF); 1 μg/ml leupeptin, 2 mM Na_3VO_4 and 10 mM NaF] for 20 min on ice. Cell debris was removed by centrifugation at 10 000 × g for 10 min at 4 °C. JNK was immunoprecipitated with 0.5 μg of a rabbit polyclonal anti-JNK antiserum (Santa Cruz Biotechnology) and 20 μl protein A-Sepharose beads for 2 h. Beads were washed three times with kinase lysis buffer and twice in assay buffer (20 mM MOPS, pH 7.2; EGTA 10 mM; 10 mM $MgCl_2$, 0.1 % Triton X-100 and 1 mM DTT). Beads were left in

a 1:1 suspension and kinase reactions were started by addition of 0.5 μg GST-jun(1-79) and ATP (100 μM ATP and 5 μCi [γ-^{32}P]ATP). After incubation for 20 min at room temperature reactions were stopped by adding 25 μl sixfold concentrated Laemmli buffer and boiling for 5 min. Finally samples were resolved on an SDS-polyacrylamide gel, transferred to nitrocellulose and analyzed using a phosphorimager.

4.6 Western blotting

Cells were sonicated in PBS containing a mix of protease inhibitors (Complete TM, Roche Molecular Biochemicals, Rotkreuz, Switzerland) and centrifuged for 10 min at 10 000 × g. Lysates and pellets were heated (5 min, 95 °C) in PNGaseF denaturation buffer (1 % 2-ME and 0.5 % SDS), then supplemented with 1 % NP40 and 50 mM sodium phosphate pH 7.5 and digested for 1 h with or without 100 U of PNGaseF. Subsequently proteins were separated by SDS-PAGE and transferred to nitrocellulose. Apo3/TRAMP was detected with anti-TRAMP antibodies (50 ng/ml) followed by peroxidase-labeled goat anti-rabbit antibodies (1.4000 dilution, Jackson Immunoresearch, West Grove, PA).

4.7 RNase protection assays

RNA was isolated from Kym-1 cells with RNA INSTAPURE (Eurogentech, Belgium) according to the manufacturer's recommendations. Subsequently, RNase protection assays were performed with the hAPO-3c Multi-Probe template set (PharMingen, Hamburg, Germany) containing probes for caspase-8, FasL, Fas DcR1, DR3, DR4, DR5, TRAIL, TNFR1, TRADD, RIP and L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal controls. Probe synthesis, hybridization and RNase treatment were performed with the RiboQuant Multi-Probe RNase Protection Assay System (PharMingen, Hamburg, Germany) according to the manufacturer's recommendations. Samples were resolved by electrophoresis on denaturing polyacrylamide gels (5 %) and quantified by phosphorimaging.

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